EVALUATING PERFORMANCE EFFECTS OF LOW-LEVEL INHALATION EXPOSURE TO NERVE AGENTS IN RATS

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1. Abstract

To investigate cognitive and general performance effects of low-level exposure to an organophosphorus chemical warfare agents (CWA), we evaluated behavior in rats inhalation-exposed (whole body, 60 min duration) to GB (1.7 mg/m3 – 4.0 mg/m3 X 1; 4.0 mg/m3 x 3) and GF (1.6-5.2 mg/m^3 X 1). Before exposure, rats were trained on a variable-interval 56 sec schedule of reinforcement (VI56), which served as a general index of behavioral performance. Beginning 48 hours after inhalation exposure, testing on the VI56 continued and acquisition of a four-of-eight, radial-arm maze task began. Performance on the maze task provided a measure of acquisition of a new task having a substantial cognitive component (i.e., spatial memory). Evaluations using both procedures were conducted during 55 sessions occurring during approximately 11 weeks following exposure. Performance deficits were observed, as a decrease in response rate, on the VI56 procedure following the highest concentrations tested of GB and GF. The deficits, however, were small and resolved relatively quickly. Small differences in the initial phases of acquisition on the maze task were observed in that some CWA-exposed rats tended to take longer to solve the maze and to make more errors. All rats, however, learned and maintained proficiency on the task. No deficits were observed to have a delayed onset. These results demonstrate that, in rats, low-level, largely asymptomatic, inhalation exposure to CWA can produce small performance deficits, but the deficits are not persistent.

2. Introduction

Near-term effects of high-dose exposure to organophosphorus chemical warfare nerve agents (CWA) are relatively well documented. The agents are extremely potent and rapidly inhibit acetylcholinesterase. The resulting increase in cholinergic activity produces a cascade of detrimental effects that, when exposure is sufficient, are lethal. The effects of low-level exposures have not been investigated to the same extent. That is, research gaps exist in our understanding of the toxicological effects of low-level exposure to CWA and further information is useful to evaluate risk assessment, health effects, and operational impact issues. In this regard, a "low-level" exposure is generally regarded as one that is asymptomatic or produces only mild symptoms without producing overt signs of clinical toxicity such as convulsions. Three integral aspects of our interest in low-level exposure to CWA are to: 1) establish the minimum exposure that produces a performance deficit, 2) characterize the recovery from exposures that produce only mild symptoms, and 3) evaluate the possibility that exposures, not producing immediate clinical signs of toxicity, could subsequently produce performance and cognitive deficits.

To evaluate cognitive and general performance effects, we used the established behavioral techniques of operant conditioning and radial-arm maze learning. Both of these procedures have been shown to be sensitive to the effects of a wide range of pharmacological and chemical agents, including cholinesterase inhibitors (Genovese & Doctor, 1997; Genovese, et al., 1996; 1993; Olton, 1987; Walsh, & Chroback. 1987). We employed a design to measure effects on previously learned behavior (i.e., by comparing performance before and after exposures), as well as on the acquisition of new behaviors (i.e., comparing learning in exposed and unexposed animals). To evaluate the potential for moderately long-term and delayed effects, performance was evaluated over an approximately 11-week period following exposure.

3. Methods

3.1 Animals

Adult male Sprague-Dawley rats, individually housed in a temperature-controlled environment under a 12L: 12D cycle were used. Water was always available in the home cages and body weights were maintained at approximately 320 g by food administered during experimental sessions and supplemental feedings.

3.2 Variable interval schedule of reinforcement

Sessions were conducted in ten standard rodent operant conditioning chambers housed in ventilated, light-

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and sound-attenuating cubicles. Each chamber contained two response levers and a food trough attached to a food dispenser capable of delivering 45-mg food pellets. Each chamber also contained a houselight, mounted on the front wall, and a stimulus light panel mounted above each of the response levers. Experimental events were controlled and monitored by a Pentium® microcomputer, using the Med-PC® control software (Med Associates).

Rats were initially trained to lever-press for food pellets under a continuous schedule of reinforcement. Although two levers were present in each chamber, only one lever produced food reinforcement. When lever pressing was maintained by food presentation, rats were trained to lever-press under a variable-interval, 56 sec (VI56) schedule of food reinforcement. The schedule specifies that a single lever-press, following an average interval of 56 sec, produces food reinforcement (i.e., a single food pellet). Interval values for the schedule were chosen pseudorandomly, without replacement, from normal distributions (range=2.44-198.23 sec) The houselight and the stimulus lights above both levers were illuminated during the sessions. Sessions were 30 min in duration. When responding under the schedule of reinforcement was stable, rats were assigned to treatment groups matched with respect to rate of responding.

3. 3 Radial-Arm maze

Maze sessions were conducted using an eight-arm, radial-maze measuring 137.2 cm in diameter. The center of the maze was a plastic octagon hub measuring 26.67 cm across, with a Plexiglas lid and wire grid floor. A Plexiglas arm with a metal bar floor was attached to each of the eight sides of the hub. Each arm's runway contained two floor-mounted switches which were depressed by the weight of the rat when present in the proximal and distal portion of the runway, respectively. The terminal portion of each arm contained a food trough outfitted with a photo-emitter / detector unit which detected access by the rats. Breaking the beam was considered a choice response. The terminal portion also contained a pellet dispenser for delivering 45-mg food pellets. Experiments were controlled and monitored with a Pentium® microcomputer using the L2T2S software control system (Coulbourn Instruments).

Rats were trained on a four-of-eight maze task in which four of the arms are baited (i.e., a nose-poke in the food trough of a baited arm could produce a single food pellet). The same set of baited arms was maintained for individual rats, but could vary between rats and was assigned randomly from a set of 37 possible configurations having no more than two consecutive arms baited. A single pellet was available in each baited arm in a single session. The session was terminated when a rat had visited all baited arms or 15 min had elapsed. No familiarity training with the maze was conducted prior to the first session.

3.4 Agent exposure

Chemical agent standard analytical reagent material (CASARM)-grade isopropyl methylphosphonofluoridate (GB) (lot # GB-U-6814-CTF-N (GB2035)) was verified as 98.3 ± 0.48 wt. % pure (as determined by quantitative NMR 31P) and stored in sealed ampoules containing nitrogen. Ampoules were opened as needed to prepare external standards or to be used as neat agent for vapor dissemination. Cyclohexyl methylphosphonofluoridate (GF or cyclosarin) (lot # GF-93-0034-109 (GF-S-6092-CTF-N-1)) was distilled by ECBC's Advanced Chemistry Team and verified as 98.87 ± 0.50 wt. % pure as determined by quantitative NMR 31P (Brickhouse, et al., 1997) and stored in sealed ampoules containing nitrogen. Ampoules were opened as needed to prepare external standards or to be used as neat agent for vapor dissemination.

Rat whole body inhalation exposures to GB or GF vapor or air control were conducted by the Operational Toxicology Team, Research and Technology Directorate at the US Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, Maryland. Exposures were conducted in a 750 L dynamic airflow chamber constructed of stainless steel with Plexiglas windows on each of its six sides. Control rats were exposed to air only in a separate chamber identical in construction to the agent chamber. All exposures were 60 min in duration and rats were placed in stainless steel compartmentalized cages (20" w x 14" l x 4" h) during the exposure.

Rats were exposed to vapor generated by two methods, depending upon the concentration required. For higher concentrations, the vapor generation system consisted of a gas-tight syringe (Hamilton, Reno, NV), variable-rate syringe drive (Model 22, Harvard Apparatus Inc., South Natick, MA), and other vaporization equipment (see Anthony, et al., 2004; Mioduszewski, et al., 2002). For lower concentrations, saturated vapor streams were generated by directing nitrogen carrier gas through the inlet of a glass vessel containing liquid agent. The glass vessel (saturator cell) consisted of a 100-mm long, 25-mm o.d. cylindrical glass tube with two vertical 7-mm o.d. tubes connected at each end (inlet and outlet tubes). The cylindrical tube of the saturator cell contained a hollow ceramic cylinder that served to increase the contact area between the liquid agent and the nitrogen. The saturator cell was fabricated to allow nitrogen to make three passes along the surface of the wetted ceramic cylinder before exiting the outlet arm of the saturator cell. The saturator cell body was immersed in a constant temperature bath so that a combination of nitrogen flow and temperature could regulate the amount of GF vapor going into the inhalation chamber.

Two sampling methods were used to monitor and analyze the GB and GF vapor concentration in the exposure chamber. The first method was a quantitative technique using solid sorbent tubes (Tenax/Haysep) to trap GB or GF vapor, followed by thermal desorption and gas chromatographic (GC) analysis (HP Model 6890, Agilent Technology, Baltimore, MD). The second method was a continuous monitoring technique using a phosphorus monitor (HYFED, Model PH262, Columbia Scientific, Austin, TX) (see Anthony, et al., 2004; Mioduszewski, et al., 2002).

3.5 Agent regeneration assay

Post-exposure blood samples were taken from the tail of all rats approximately 30 min following the end of exposure. Plasma and red blood cell (RBC) fractions were assayed for GF using mass spectrometry (GC/MS) (see Jakubowski, et al., 2002, for procedure details).

3.6 Data collection and analysis

For the VI56 sec schedule, the number of responses and the pellets earned per session were recorded. From these data, the rate of responding (responses per minute) was calculated for each lever for each rat during each session. Response rate data from ten control sessions immediately prior to exposure were averaged and subsequent response rate data (i.e., from post- exposure sessions) are expressed as a percentage of the average values obtained during control sessions for each rat (i.e., percent of control).

For the maze procedure, the total time (minutes) required to complete the maze was recorded as well as the number of errors. Additionally, errors were further differentiated into two types, reference and working. Reference errors occurred if an arm was visited that was not baited. Working errors occurred if a baited arm was revisited more than once during the session.

Inferential statistics were calculated using the SAS (Cary, NC) statistical software package. For within-groups effects, a MANOVA (Wilk's Lambda) or ANOVA was used. Between-groups effects were assessed with ANOVA. Multiple contrasts comparing sarin-exposed groups with air control groups were evaluated with two-tailed Dunnett's t-tests.

4. Results

Rats learned to respond for food on the VI56 sec schedule with a relatively constant rate of responding during the 30-min sessions. When responding was stable, rats were assigned to treatment conditions (n=5 or 6, each group) balanced with respect to baseline response rates. Responding on the lever that did not produce food (inactive lever) was low in all rats. Typically, only a few responses were made on the inactive lever during a session. That is, the number of responses on the inactive lever was always a small percentage of the total number of responses. No systematic changes in responding on the inactive lever were observed under any treatment conditions.

All rats were observed within 60 min following the completion of inhalation exposures for the presence of the following signs of organophosphorus toxicity: miosis, ataxia, tremors, subconvulsive jerks, convulsions, salivation, straub tail, exophthalmos, gasping, collapse, prostration and unstable gait. In general, all GB- and GF-exposed rats were observed to have some degree of miosis. At the higher concentrations, this included pinpoint pupils. Other clinical signs of OP intoxication were generally not present except following the highest concentrations examined.

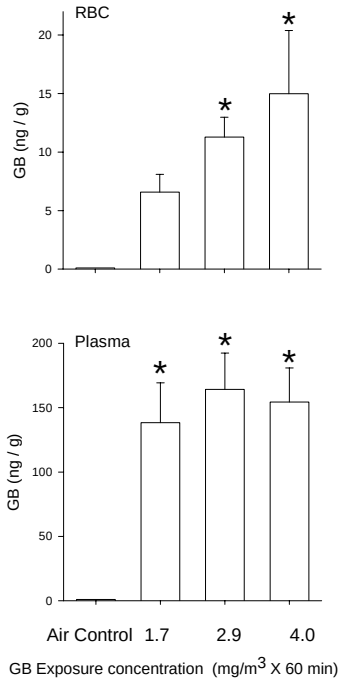

Figure 1. Regenerated sarin in RBC (top) and plasma (bottom) in rats after inhalation exposure to GB or air control. Each bar represents the mean (± SEM) from six rats. Blood was sampled at approximately 30 min after the end of the exposure.

Blood sampled following exposure revealed statistically significant and dose-dependent regenerated GB and GF (see Figures 1 and 2). Regenerated agent (GB and GF) in RBC fractions (Figures 1 and 2, top) was substantially less than that found in plasma (Figures 1 and 2, bottom panels). In this regard, plasma agent (GB and GF) levels were 12-25 fold more than RBC levels. The difference likely reflects GB and GF inhibition of acetylcholinesterase in RBC versus inhibition of butyrylcholinesterase and carboxylesterase in plasma. As

expected, no GB or GF was found in any samples from the air control treated rats. These results suggest that the regeneration assay is a good indicator of systemic exposure and may be particularly useful for comparing exposure magnitude across species and routes of administration.

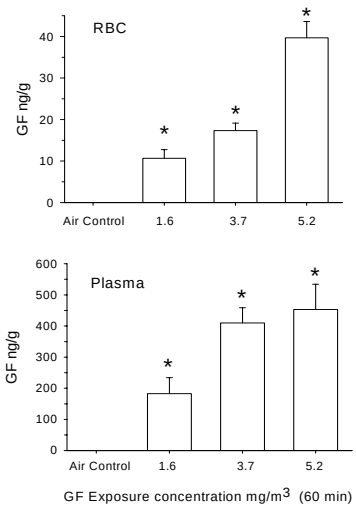

Figure 2. Regenerated GF in RBC (top) and plasma (bottom) in rats after inhalation exposure to GF or air control. Each bar represents the mean (± SEM) from six rats. Blood was sampled approximately 30 min after the end of the 60 min exposure.

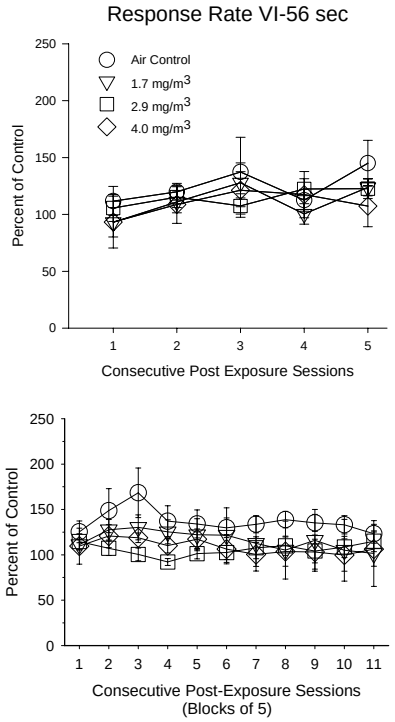

Figure 3. Rate of responding under the VI56 sec schedule of reinforcement in rats following exposure to GB or air control. Top: Responding during the five consecutive sessions following exposure. Bottom: Responding during the 55 sessions following exposure (in blocks of five consecutive sessions). Response rate is expressed as a percentage of the average response rate during the ten sessions preceding exposure (i.e., control). Each point represents the mean (+/- SEM) from six rats.

Figures 3 and 4 show response rate data characterizing performance on the VI56 task during the first five post-exposure sessions (top) and the 55 post-exposure sessions, as blocks of five sessions (bottom) following single GB (Figure3) and GF (Figure 4) exposures. For data from the first five post-exposure sessions, only the largest concentration of GF (5.2 mg/m$^{3)}$ produced a significant deficit. Further, disruption was only observed during the initial sessions of the block. Therefore, the largest concentration of GF produced a small performance deficit on the VI56, but the deficit was not persistent as recovery was rapid. None of the single exposures to GB produced significant effects on the VI56. In general, no deficits were observed for blocks of sessions over the entire post-exposure period (55 sessions) in either GB or GF exposed groups. Thus, long term responding on the VI56 was unaffected by any concentration of GF, and we observed no evidence that would suggest a delayed deficit.

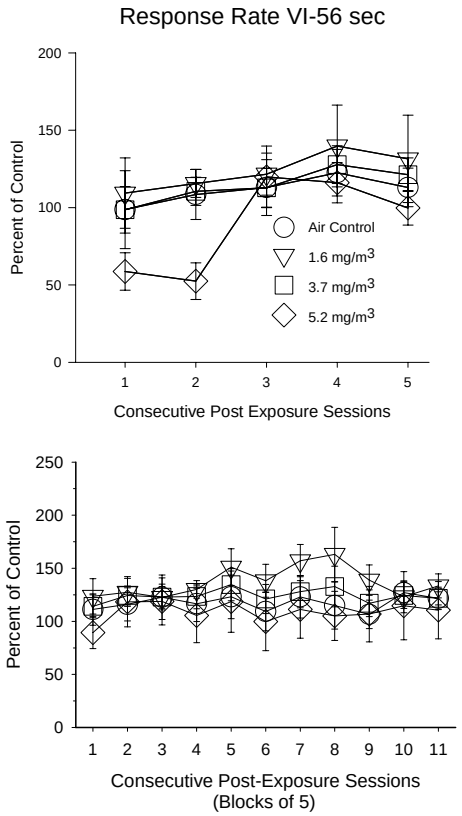

Figure 4. Rate of responding under the VI56 sec schedule of reinforcement in rats following exposure to GB or air control. Top: Responding during the five consecutive sessions following exposure. Bottom: Responding during the 55 sessions following exposure. Response rate is expressed as a percentage of the average response rate during the ten sessions preceding exposure (i.e., control). Each point represents the mean (+/- SEM) from six rats.

Figure 5 shows response rate data characterizing performance on the VI56 task during the first five post-exposure sessions (top) and the 55 post-exposure sessions, as blocks of five sessions (bottom) following three consecutive daily exposures to GB (4.0 mg/m^3). Unlike with single exposures to GB, this treatment produced a response deficit during the initial sessions conducted after exposure. The deficit, however, was not persistent as recovery was rapid. No deficit was observed for blocks of sessions over the entire post-exposure period (55 sessions). Thus, long-term performance on the VI56 was unaffected by the multiple exposure treatment, and we observed no evidence that would suggest a delayed deficit.

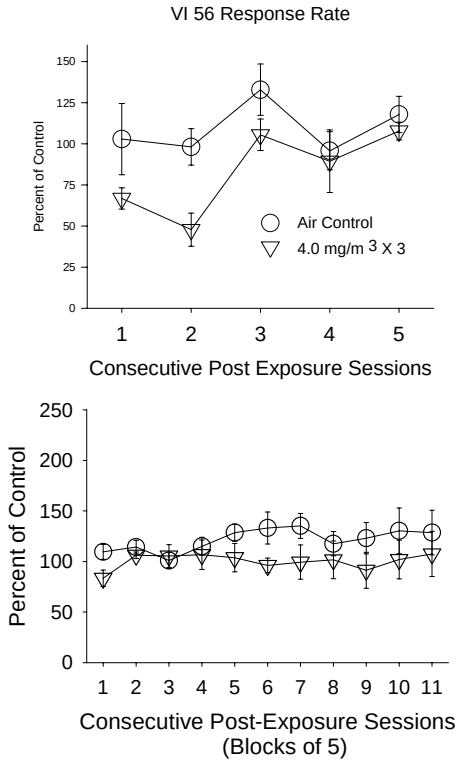

Figure 5. Rate of responding under the VI56 sec schedule of reinforcement in rats following three daily exposures to GB or air control. Top: Responding during the five consecutive sessions following exposure. Bottom: Responding during the 55 sessions following exposure. Response rate is expressed as a percentage of the average response rate during the ten sessions preceding exposure (i.e., control).Each point represents the mean (+/- SEM) from five rats.

Following exposures, all rats were trained on the radial-maze task. Figures 6-8 show the measures of completion time (top) and total errors (bottom) during the 55 post-exposure sessions following single (Figure 6) and multiple (Figure 8) exposures to GB and single (Figure 7) exposures to GF. Acquisition of the task was systematic and all rats learned the task to a reasonable level of accuracy (i.e., rats learned to make progressively fewer errors on the maze and to complete the task in a progressively shorter time).

At the largest concentration, single exposures to GB tended to increase the time to complete the maze during the initial sessions (i.e., Figure 6, top, block 1), neither the time by exposure interaction MANOVA nor the between groups ANOVA for block 1 was statistically significant. Similarly, rats exposed to GF tended to take longer to complete the maze during the beginning of acquisition (i.e., Figure 7, top, block 1), neither the time by exposure interaction MANOVA nor the between groups ANOVA for block 1 was statistically significant. Thus, single exposures to GB or GF did not significantly effect completion time on the maze task. Multiple exposures to GB (Figure 8, top), however, did increase the time to complete the maze as evidenced by a significant treatment by session interaction and significant between-groups comparisons. The effect was only observed during the early portion acquisition.

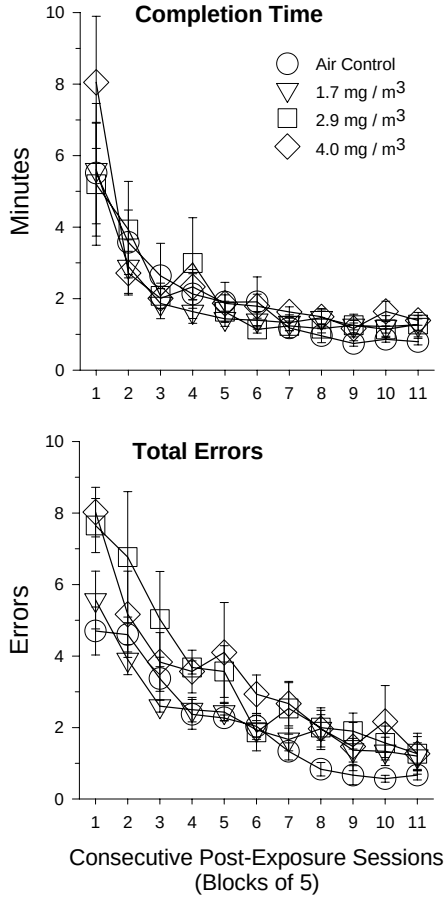

Figure 6. Average completion time (top) and number of errors (bottom) on the radial-arm maze task in rats during 55 sessions (in blocks of 5 consecutive sessions) following inhalation exposure to GB or air control. Each point represents the mean (± SEM) of six rats.

The higher concentration of GB resulted in an increase in errors during the early phase of acquisition. Single exposures to GB at the larger concentrations (Figure 6, bottom) showed significant differences from control at block 1. Similarly, the multiple exposures to GB (Figure 8, bottom) produced significant difference in errors from control during block 2. None of the between groups comparisons for the 11 blocks of sessions for GF, however were significant (Figure 7, bottom). Thus, GB produced small decreases in accuracy at higher concentrations. GF, at the concentrations tested, did not affect accuracy on the maze task.

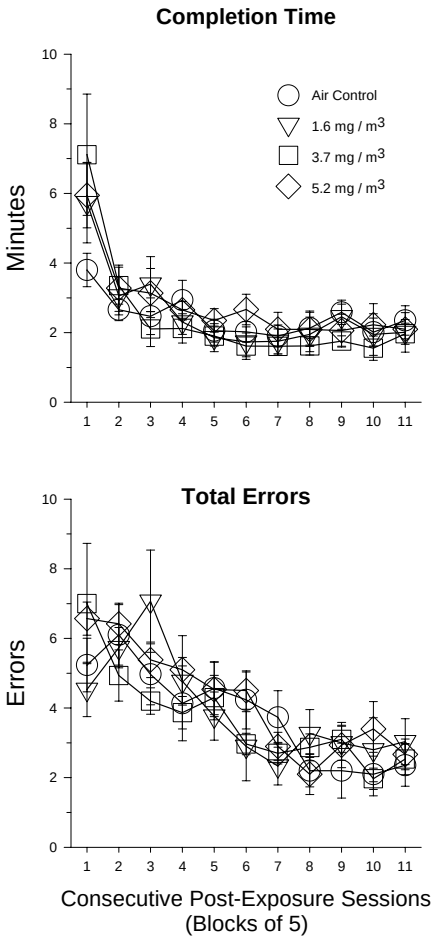

Figure 7. Average completion time (top) and number of errors (bottom) on the radial-arm maze task in rats during 55 sessions (in blocks of 5 consecutive sessions) following inhalation exposure to GF or air control. Each point represents the mean (± SEM) of six rats.

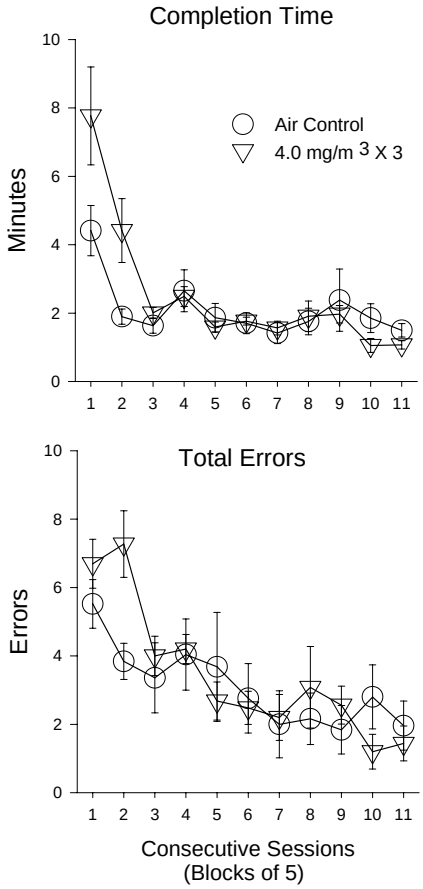

Figure 8. Average completion time (top) and number of errors (bottom) on the radial-arm maze task in rats during 55 sessions (in blocks of 5 consecutive sessions) following three consecutive daily inhalation exposures to GB or air control. Each point represents the mean (± SEM) of five rats.

5. Discussion

Whole-body inhalation exposures to GB (1.7 mg/m3 – 4.0 mg/m3 X 1; 4.0 mg/m3 x 3, 60 min duration) and GF (1.6-5.2 mg/m3 X 1, 60 min duration) were evaluated in rats for behavioral effects on a previously learned task (VI56) and the acquisition of a new task (radial-arm maze). Blood sampled following exposures showed dose-dependent, regenerated GB and GF, and substantially more of each agent was found in plasma than in RBC fractions. The systematic regeneration of agent serves as a verification of systemic exposure.

Exposures produced temporary miosis in all rats and mild symptoms at only the highest concentrations. None of the exposures produced severe symptoms such as convulsions. Larger concentrations than those tested in the present report were found, in our laboratory, to result in convulsions and, in some cases, lethality. Thus, the largest concentrations used here are probably near the threshold for producing major clinical symptoms.

Small deficits on a previously learned VI56 task were observed following the largest concentrations of GB and GF examined. The deficits were seen as a decrease in the rate of responding on the task, but did not disrupt acquisition of the radial-maze task. The performance deficits were not persistent and recovery was observed within a few sessions. That is, responding returned to pre-exposure levels. No deficits had a delayed onset during the course of 55 test sessions occurring over approximately 11 weeks following exposure.

All rats acquired and maintained performance on the radial-maze task. Small differences in acquisition were, however, observed with rats following exposure to the higher concentrations (e.g., multiple GB exposures). The differences were observed as an increase in errors and in increase in time required to complete the maze. The deficits occurred early in acquisition (i.e., during the first weeks of acquisition training). Further, the deficits did not persist as all rats learned the maze to the same general performance level.

Taken together, these results suggest that low-level and largely asymptomatic (i.e. non-convulsive) exposures to CWA can produce small cognitive and general performance deficits in rats. The deficits were observed to be small and recovery was relatively rapid and complete. Further, no evidence of a delayed onset was observed.

6. Footnote

The views of the authors do not purport to reflect the position of the Department of the Army or the Department of Defense, (para 4-3, AR 360-5). Research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 edition. All procedures were reviewed and approved by the Institutes' Animal Care and Use Committees, and performed in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

7. Acknowledgements

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